Food Chemistry 197 (2016) 1235–1239

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Water electrolyte promoted oxidation of functional thiol groups

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ARTICLE INFO

Article history:
Received 26 June 2015
Received in revised form 12 November 2015
Accepted 13 November 2015
Available online 14 November 2015

Keywords:
Thiol oxidation
Disulfide cross-linking
Glutathione
Cysteine
Water electrolyte composition
$CaCl_2$
Gluten dough

ABSTRACT

The formation of disulfide bonds is of the utmost importance for a wide range of food products with gluten or globular proteins as functional agents. Here, the impact of mineral electrolyte composition of aqueous solutions on thiol oxidation kinetics was studied, using glutathione (GSH) and cysteine (CYS) as model systems. Interestingly, the oxidation rate of both compounds into their corresponding disulfides was significantly higher in common tap water than in ultrapure water. The systematic study of different electrolyte components showed that especially $CaCl_2$ improved the oxidation rate of GSH. However, this effect was not observed for CYS, which indicated a strong impact of the local chemical environment on thiol oxidation kinetics.

1. Introduction

Organosulfur compounds carrying functional thiol groups (RSH) or their oxidized disulfide bridge linked dimers (RSSR) play a crucial role in biochemistry and pharmaceutical, chemical and food industries (Chatterjee & Ranu, 2013). Thiol oxidation and thiol/disulfide interchange reactions in and between gluten proteins during wheat dough fermentation and bread baking impact dough processability and final bread quality (Lagrain, Thewissen, Brijs, & Delcour, 2008). Even small variations in thiol/disulfide ratios have a large impact on dough rheology and corresponding bread loaf volume (Jones, Phillips, & Hird, 1974).

Gel texture from globular proteins also depends on oxidation of functional thiols to inter- and intramolecular disulfides (Visschers & De Jongh, 2005). For instance, the functionality of whey protein gels was linked to the number of thiol groups, and larger disulfide cross-linked protein structures were correlated to increased gel hardness (Alting, Hamer, De Kruif, Paques, & Visschers, 2003). The importance of disulfide bonds has been demonstrated for gels from a wide range of food albumins and globulins including hen egg ovalbumin (Kitabatake, Hatta, & Doi, 1987). Furthermore, the conversion of wheat gluten proteins into bioplastics also relies on disulfide cross-linking. These sustainable low-cost alternatives to petroleum derived polymers offer good mechanical and rheological properties, superior biodegradability and low toxicity (Jansens et al., 2014; Lagrain, Goderis, Brijs, & Delcour, 2010). Thiol redox kinetics are also important for disulfide-cleavage-triggered drug delivery systems (Lee et al., 2013), and regeneration of certain self-healing polymer films through thiol/disulfide redox exchange reactions (Yoon et al., 2012).

Several studies have reported the influence of mineral salts on wheat flour dough rheology. While it is assumed that salts shield the charges of proteins, thereby reducing electrostatic repulsion and allowing proteins to associate and produce a stronger dough, this theory does not explain all results (Tuhumury, Small, & Day, 2014). For instance, effects depend on concentration as well as the type of salts. Remarkably, few if any studies investigated the possible impact of electrolytes on thiol oxidation kinetics. Increased insight into water electrolyte promoted thiol oxidation is of high societal relevance and can be expected to significantly influence industrial production streams.

This work studied the impact of the electrolyte composition on the oxidation activity of functional thiol groups for two organosulfur model compounds, glutathione (GSH) and cysteine (CYS). GSH is a tripeptide (γ-glutamylcysteinylglycine) which contains the amino acid CYS. Both compounds can be oxidized into their corresponding disulfides, glutathione disulfide and cystine, respectively. The oxidation of GSH and CYS was studied in the presence of an additional oxidant $KBrO_3$ under mild conditions (30 °C, pH 6.5) in ultrapure water, in tap water and in different electrolyte solution series to investigate the effect of the mineral electrolyte

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http://dx.doi.org/10.1016/j.foodchem.2015.11.075
0308-8146/

composition in tap water. $KBrO_3$ was a popular additive in the bread making industry. It oxidizes free thiol groups of low molecular weight peptides to disulfide compounds thereby enhancing gluten functionality (Joye, Lagrain, & Delcour, 2009).

2. Materials and methods

2.1. Materials

$Ca(NO_3)_2$ (99%), $Mg(NO_3)_2$ (99%), $NaNO_3$ (99%), $CaCl_2$ (99%), $CaSO_4$ (99%), $MgCl_2$ (99%), L-glutathione reduced, L-cysteine, DTNB, $KBrO_3$ and MOPS were purchased from Sigma–Aldrich (Steinheim, Germany); NaCl (99%) from VWR International (Leuven, Belgium) and $CaCO_3$ (99%) from Acros Organics (Geel, Belgium). Ultrapure water (MilliQ, minimum resistivity 18.2 MΩ) was used to prepare the mineral electrolyte solution series.

2.2. GSH and CYS model systems

First, MOPS buffers (25 mmol/L; pH 6.5) were prepared from ultrapure water, tap water and different mineral electrolyte solution series. MOPS buffer has negligible metal ion binding affinity, is typically used in biochemical and biological research and has an effective pH range of 6.5–8.0. Based on the mineral composition of tap water in Leuven, Belgium (Table 1), following electrolyte solution series were selected: a nitrate solution series [$Ca(NO_3)_2$; $Mg(NO_3)_2$; $NaNO_3$], a calcium solution series [$CaCl_2$; $CaSO_4$; $Ca(NO_3)_2$; $CaCO_3$] and a chloride solution series [$CaCl_2$; $MgCl_2$; NaCl]. Ions in tap water with concentrations lower than 0.1 mmol/L were not considered. Carbonate (CO_3^{2-}) was included in the experiments because its concentration in tap water can be expected to be in equilibrium with CO_2 in the air. Then, MOPS buffers containing 0.125 mmol/L GSH or CYS, and 0 or 0.055 mmol/L $KBrO_3$, were shaken and heated for 24 h at 30 °C. All experiments were performed in triplicate. The concentrations of monovalent and divalent cation salts were 8 mmol/L and 4 mmol/L in the reaction medium, respectively.

Table 1
Mineral ion composition in tap water in Leuven, Belgium (De Watergroep).

Ion	Average concentration (mmol/L)
Calcium (Ca^{2+})	3.08
Magnesium (Mg^{2+})	0.49
Sodium (Na^{+})	0.87
Chloride (Cl^{-})	1.44
Sulfate (SO_4^{2-})	0.51
Nitrate (NO_3^{-})	0.83

2.3. Free SH determination

The concentration of GSH or CYS in solution was determined by colorimetric analysis of the free thiol content after reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). 750 μL of a DTNB solution [0.26 mmol/L DTNB and 2.0 mmol/L EDTA in MOPS buffer (250 mmol/L; pH 8.0)] was added to 750 μL of the sample solution. The mixtures were shaken and extinction at 412 nm was determined exactly 8 min after adding the DTNB solution. Absorbance values were converted to the concentration of free thiol groups using a calibration curve with GSH. Control solutions containing either no DTNB or no sample were used to correct for background extinction of DTNB and sample (Lagrain, Brijs, Veraverbeke, & Delcour, 2005).

3. Results and discussion

3.1. Impact of tap water on GSH and CYS oxidation

Compared to the reference system prepared in ultrapure water, the use of tap water accelerated thiol oxidation kinetics of both GSH and CYS. In the reference system after 24 h, 32% and 75% of the initial GSH concentration remained unoxidized in presence and absence of $KBrO_3$, respectively. In tap water, GSH was fully oxidized after 24 h, either with or without $KBrO_3$ (Fig. 1A). In the presence of $KBrO_3$, CYS was already completely oxidized after 2 h in tap water, while this was only the case after 24 h in ultrapure water (Fig. 1B).

CYS was found more reactive towards oxidation than GSH. In ultrapure water with $KBrO_3$, CYS was fully oxidized after 24 h while 32% of GSH remained unoxidized. In tap water with $KBrO_3$, CYS and GSH oxidation were complete after 2 h and 24 h, respectively (Table S1).

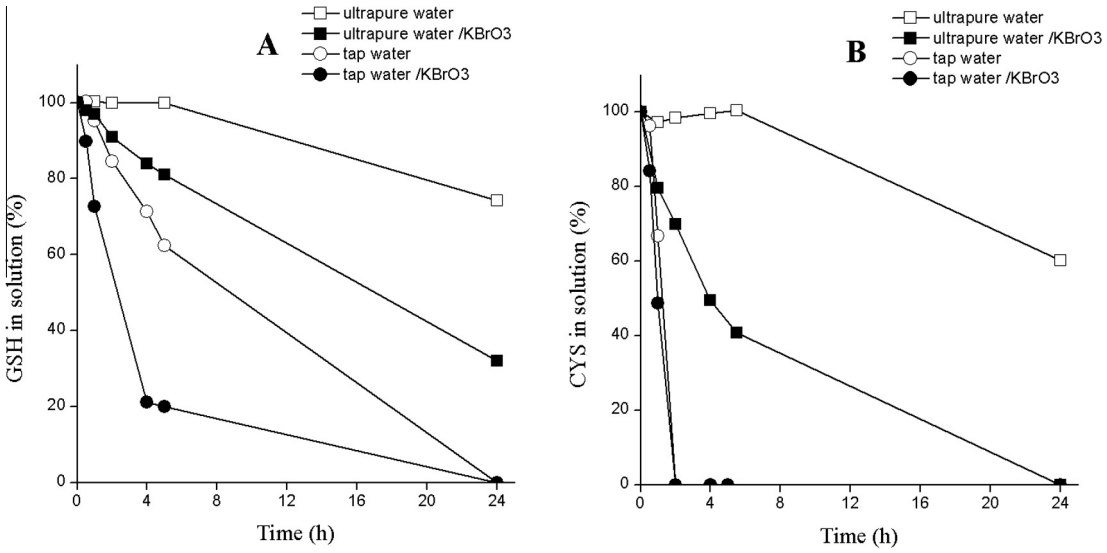

Fig. 1. The oxidation of GSH (A) and CYS (B) in ultrapure water, with and without $KBrO_3$.

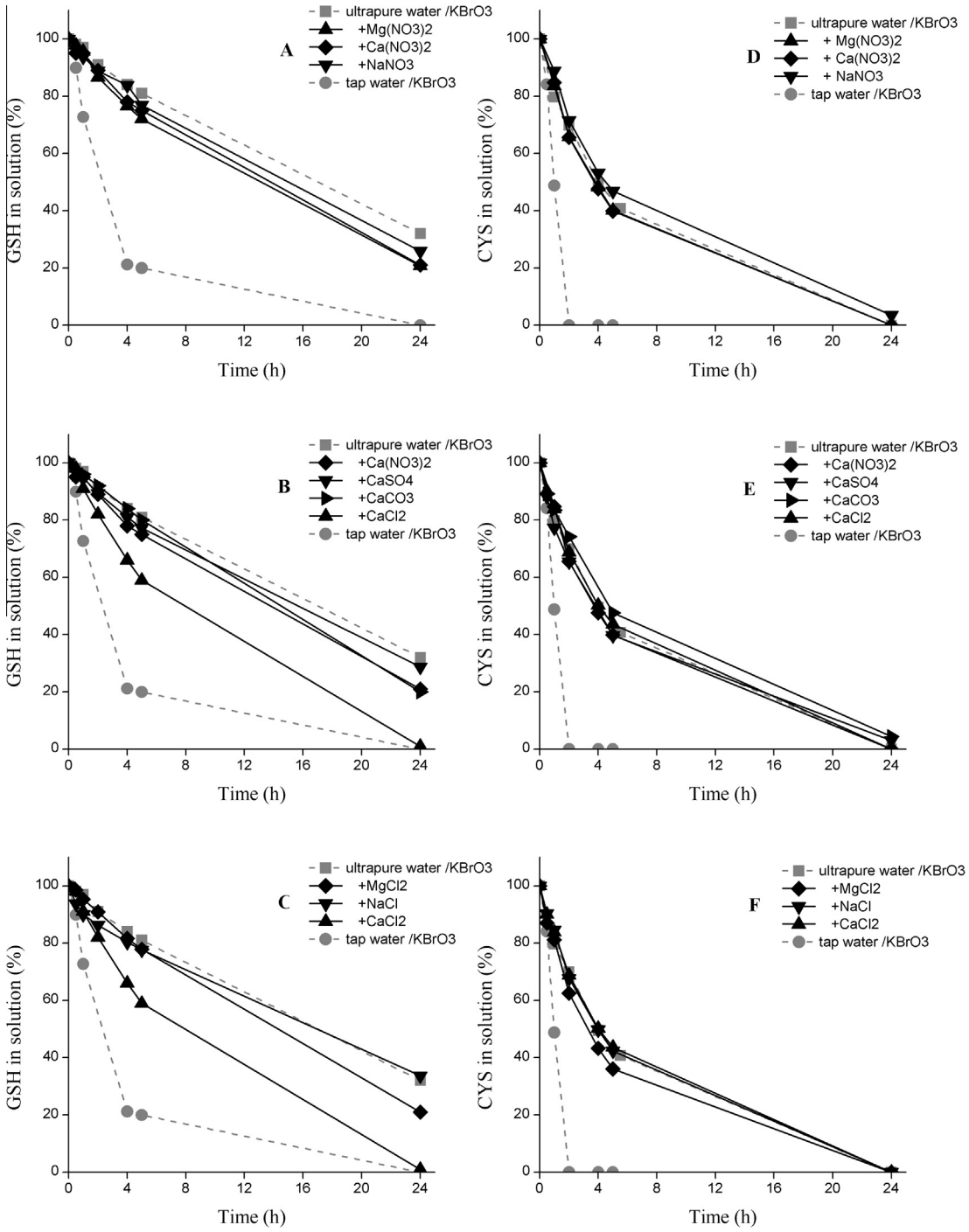

Fig. 2. The oxidation of GSH (A–C) and CYS (D–F) in presence of $KBrO_3$ and nitrate (A and D), calcium (B and E) and chloride (C and F) electrolytes. Grey dotted lines represent the oxidation of GSH (A–C) and CYS (D–F) in presence of $KBrO_3$ in ultrapure and tap water.

3.2. Impact of electrolytes on GSH and CYS oxidation

The oxidation of GSH and CYS in ultrapure water were monitored in presence of $KBrO_3$ and different electrolytes, commonly present in tap water (Table S1). Electrolytes of the nitrate salt series [$NaNO_3$, $Mg(NO_3)_2$, $Ca(NO_3)_2$] did not impact the oxidation rate of GSH (Fig. 2A). After 24 h, 20% to 30% of the initial GSH concentration remained in solution, displaying similar oxidation kinetics as the reference system containing $KBrO_3$ in ultrapure water. The same observation was made for most electrolytes of the calcium series [$Ca(SO_4)$, $Ca(CO_3)$, $Ca(NO_3)_2$], except for $CaCl_2$ (Fig. 2B). $CaCl_2$ significantly enhanced the oxidation kinetics of GSH, leading to complete oxidation of GSH after 24 h. The prominent impact of $CaCl_2$ was not observed for the other chloride electrolytes $MgCl_2$ or NaCl (Fig. 2C). Remarkably, no electrolyte impacted CYS oxidation kinetics, not even $CaCl_2$ (Fig. 2D–F).

All standard deviations were lower than 5%, except for CYS in ultrapure water without $KBrO_3$ (standard deviation = 42%). Thiol oxidation in the latter is only impacted by the dissolved oxygen content, which can slightly vary depending on time of use of the ultrapure water. It is however clear that the effect of tap water itself and the additional oxidant $KBrO_3$ on thiol oxidation of both

GSH and CYS outweighs that of dissolved oxygen and that the effect of such differences in dissolved oxygen content can be neglected in presence of the oxidant $KBrO_3$.

3.3. Discussion

In presence of $KBrO_3$, thiol oxidation of CYS proceeded faster than that of GSH, both in ultrapure and tap water. These results were in agreement with reports by Hird and Yates (1961) on the oxidation of GSH and CYS in ultrapure water at pH 6.0 and 28 °C, also in presence of $KBrO_3$ (thiol/bromate ratio = 1). There, 80% of CYS was oxidized after 1 h as compared to only 50% of GSH. The oxidation rate of the thiol groups in CYS and GSH has been related to their pK_a value, with higher rate constants for lower thiol pK_a values (Gough, Gargano, Donofrio, & Lees, 2003). As the pK_a of the thiol group is 8.2 in CYS and estimated to be around 8.8–9.1 in GSH, both datasets concur with that statement (Conte & Carroll, 2013; Ison, Odeh, & Margerum, 2006). Furthermore, CYS is a zwitterion in near neutral conditions (Quesada-Moreno, Avilés-Moreno, Márquez-García, & López-González, 2014), thus with no net negative charge. The possible net charge for GSH is determined by four functional groups. It has one free amino group (pK_a = 8.6; predominantly protonated = +1), two free carboxyl groups (pK_a = 3.53 and 2.12; predominantly deprotonated = −2), and the thiol group (pK_a = 9.2; predominantly non-dissociated = 0). Hence, GSH is negatively charged at near neutral pH (Lash, 2006). Obviously, the electrostatic repulsion of negatively charged GSH can play an important role in the different oxidation reactivity between GSH and CYS at near neutral pH. In addition, the GSH and CYS molecular structures (Fig. 3) also suggest that the carboxyl group of the glycine residue of GSH, which is negatively charged at pH 6.5, can cause electrostatic repulsion and steric hindrance when two diffusing GSH molecules attempt to form a disulfide bridge.

Here for the first time, a reproducible strong positive impact of common tap water versus ultrapure water on thiol oxidation activity of GSH and CYS was observed. Even without the additional oxidant $KBrO_3$, tap water itself influenced thiol oxidation strongly since CYS and GSH were already completely oxidized after 2 h and 24 h, respectively.

Evaluating the effect of various common tap water electrolytes on thiol oxidation activity showed that only $CaCl_2$ and no other electrolyte enhanced the thiol oxidation activity of GSH. The importance of $CaCl_2$ for mechanical properties of protein based bioplastic films has already been attributed to enhanced protein crosslinking due to shielding of negative charges of vicinal thiolate groups by Ca^{2+} (Gennadios, Weller, & Testin, 1993). However, the exceptional results for $CaCl_2$ in comparison to other calcium-salts presented in this manuscript do not support this hypothesis of shielding. Although the addition of $CaCl_2$ has already been studied extensively on heat-induced whey protein aggregation (Riou, Havea, McCarthy, Watkinson, & Singh, 2011), ion-protein interactions are poorly understood on a molecular level (Mounsey & O'Kennedy, 2007). The effect of simple cations on the local secondary structure of a number of model peptide protein systems has been attributed to interactions with the backbone carbonyl groups, however, such ion-induced disruptions have not been reported for thiol containing peptides (Baldauf et al., 2013; Shi & Wang, 2014).

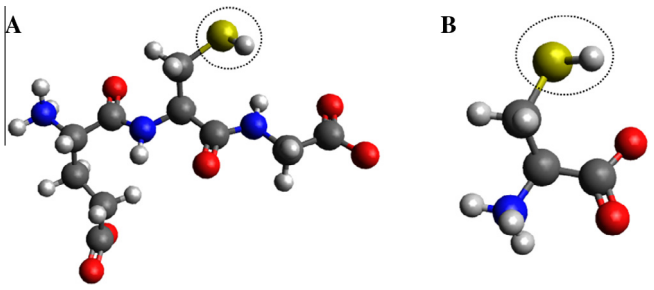

Fig. 3. The molecular structure of GSH (A) and CYS (B), created with the Avogadro program. The functional thiol group is encircled and represented by the yellow atom. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$CaCl_2$ did not impact thiol oxidation of CYS, while it impacted that of GSH. This implies an influence of the molecular environment on thiol oxidation kinetics. The important role of neighboring amino acid groups on reactivity of polypeptides has also been demonstrated for other reactions. For instance, the spontaneous hydrolysis of proteins by cleavage of an internal peptide bond next to a CYS residue was affected by amino acid side groups and protein environment in general (Mihaylov, Parac-Vogt, & Pierloot, 2014).

Molecular modeling methods can assist to unravel the chemical interactions of mineral electrolyte ions with different functional thiol groups that enhance the oxidation mechanism on a molecular level (Zeida et al., 2014). While complexation reactions of metal cations with GSH (Krezel & Bal, 1999; Liu, Xia, Li, Wang, & Li, 2013; Singh, Garg, Kumar, & Singh, 2001) and CYS (Pesonen, Aksela, & Laasonen, 2010) have been analyzed extensively with different modeling methods, these theoretical studies cannot be compared easily since they investigate various types of cations using diverse assumptions. Typically, the stability order for metal thiol complexes is reported to increase with decreasing metal ion radius (Mg^{2+} > Ca^{2+}), however, none of them explicitly take into account the cation hydrated radius or presence of a counter-ion nearby.

Insights obtained during previous studies combined with experimental data presented here, indicate that the effect of $CaCl_2$ on thiol oxidation kinetics is not exclusively related to Ca^{2+} complexation. While this study clearly demonstrates the impact of common tap water and mineral electrolytes on thiol oxidation kinetics of simple model compounds, the next step would be to investigate the impact of tap water and $CaCl_2$ on the oxidation of more complex protein systems and to evaluate its effect on real food systems such as bread. Future work should also evaluate the effect of minor components and trace elements in tap water. These could also fulfill a catalytic role in thiol oxidation.

4. Conclusions

The oxidation kinetics of functional thiol groups into their corresponding disulfides were studied for two model compounds, GSH and CYS. Surprisingly, tap water enhanced the oxidation of functional thiol groups. The oxidation rates under mild conditions (30 °C, pH 6.5) of both GSH and CYS were clearly higher in tap water than in ultrapure water, both in presence and absence of the oxidant $KBrO_3$. Particularly the $CaCl_2$ electrolyte solution influenced the oxidation kinetics of GSH. However, since no pronounced effect of $CaCl_2$ was observed on the oxidation rate of CYS, it cannot be concluded that $CaCl_2$ enhances oxidation rates of functional thiol groups in general. In contrast, it implies that neighboring amino acid side groups play an important role. This highlights the importance of an in-depth analysis of the coordination chemistry of mineral ions with functional thiol groups and neighboring amino acid groups, the chemical complexation of $CaCl_2$ with GSH and the differences in interaction with CYS. A detailed assessment of the preferred relative orientation can also elucidate the differences in thiol oxidation kinetics of GSH and CYS. This study clearly demonstrated a profound but complicated

effect of tap water and mineral electrolytes on the oxidation activity of specific thiol groups. In the future, the impact of these mineral electrolytes should be identified on more complex thiol-containing food protein systems.

Acknowledgements

This research was funded by a Ph.D. grant (K.L.) from the Agency for Innovation by Science and Technology (IWT). E. Breynaert and I. Rombouts acknowledge the Research Foundation – Flanders (FWO, Brussels, Belgium) for their postdoctoral fellowship. J.A. Delcour is W.K. Kellogg Chair in Cereal Science and Nutrition at KU Leuven. J.A.D., C.E.A.K. and E.B. acknowledge long term funding in the frame of Methusalem grants (grant holders J.A. Delcour and J.A. Martens).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.11.075.

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